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TECHNICAL MANUSCRIPT 491

AMMONIUM SULFATE COPRECIPITATION  
ANTIBODY DETERMINATION WITH  
PURIFIED STAPHYLOCOCCAL ENTEROTOXINS

Jack Gruber  
George G. Wright

JANUARY 1969

DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland

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TECHNICAL MANUSCRIPT 491

AMMONIUM SULFATE COPRECIPITATION ANTIBODY DETERMINATION  
WITH PURIFIED STAPHYLOCOCCAL ENTEROTOXINS

Jack Gruber

George G. Wright

Medical Investigation Division  
MEDICAL SCIENCES LABORATORIES

Project 16061102B71A

January 1969

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

#### ABSTRACT

The ammonium sulfate coprecipitation technique of Farr was applied to purified enterotoxins of Staphylococcus aureus. Studies with iodine<sup>131</sup>-labeled enterotoxins A, B, and C, using 1.6 M ammonium sulfate for coprecipitation, revealed differences in the antigen-binding capacity of normal and immune rabbit sera for the enterotoxins. The ammonium sulfate coprecipitation procedure was more sensitive than agar gel diffusion methods for the determination of antibody to enterotoxin B. Antigen-binding tests with anti-enterotoxin B rabbit serum suggest the presence of similar antigenic determinant groups in all three toxins. Measurable antigen-binding capacities for enterotoxins A, B, and C were detected in sera of normal human subjects. Increased levels of binding antibody to enterotoxin B were detected in three of five accidentally exposed human subjects.

## CONTENTS

Abstract . . . . .	2
I. INTRODUCTION . . . . .	5
II. MATERIALS AND METHODS . . . . .	6
A. Antigens . . . . .	6
B. Antisera . . . . .	6
C. Gel Diffusion Precipitation Tests . . . . .	6
D. Labeling . . . . .	7
E. Antibody Determination . . . . .	7
F. Radioactivity Measurements . . . . .	7
III. RESULTS . . . . .	8
A. Studies with Enterotoxins A, B, and C . . . . .	8
B. Comparisons with Other Methods . . . . .	10
C. Studies with Human Sera . . . . .	14
IV. DISCUSSION . . . . .	18
Literature Cited . . . . .	21
Distribution List . . . . .	23
DD Form 1473 . . . . .	25

## FIGURES

1. Effect of Enterotoxin B Concentration on Coprecipitation with Human Serum . . . . .	9
2. Coprecipitation of Labeled Enterotoxin A and Rabbit Sera with 1.6 M Ammonium Sulfate . . . . .	11
3. Coprecipitation of Labeled Enterotoxin C and Rabbit Sera with 1.6 M Ammonium Sulfate . . . . .	12
4. Comparison Between Coprecipitation and Gel Diffusion Titers . . . .	15
5. Distribution of Coprecipitation Values Obtained with Normal Human Sera . . . . .	16

TABLES

1. Antibody to Staphylococcal Enterotoxins A, B, and C in Rabbit Sera . . . . .	13
2. Comparative Quantitative Precipitin and Ammonium Sulfate Coprecipitation Assays of Anti-Enterotoxin B Rabbit Sera . . . . .	13
3. Detection of Staphylococcal Enterotoxin B Antibody by Coprecipitation with Labeled Toxin . . . . .	13
4. Antibody to Staphylococcal Enterotoxin A, B, and C in Normal Human Subjects . . . . .	17
5. Precipitation of Labeled Staphylococcal Enterotoxin B by Sera of Exposed Human Subjects . . . . .	17

## I. INTRODUCTION

The ammonium sulfate coprecipitation technique of Farr<sup>1</sup> provides a quantitative and sensitive measurement of the antigen-binding capacity of antisera. Addition of ammonium sulfate to mixtures of iodine<sup>131</sup>-labeled antigen and antiserum precipitates the globulin, and with it the antigen that is bound to antibody; unbound antigen remains in the supernatant. The proportion of labeled antigen precipitated is a function of the antibody activity of the serum.

Farr and others have used this technique to investigate a variety of immunological phenomena, such as the production of antibody after X-irradiation;<sup>2</sup> the in vitro production of antibody following in vivo stimulation;<sup>3</sup> the kinetics of the antigen-antibody reaction;<sup>4</sup> and the types of antibody synthesized during the immune response.<sup>5,6</sup> Most investigators have used as antigen bovine serum albumin (BSA) or other stable and well characterized serum proteins. The Farr procedure was demonstrated to be more sensitive than several other established techniques for measuring antibody to bovine albumin.<sup>7</sup> Despite its sensitivity, the coprecipitation technique has been virtually neglected by those studying resistance to infection. In 1962, Freter did employ the Farr technique to detect coproantibodies against Vibrio cholerae in humans immunized by the oral and parenteral routes.<sup>8</sup> However, in this and in a later study,<sup>9</sup> partially purified "O" antigen preparations were used. Farr's method has also been used to study the immune response to streptococcal M protein<sup>10</sup> and to anthrax protective antigen.<sup>11</sup> Again, the antigens employed were semipurified.

Because of the sensitivity and precision of the ammonium sulfate coprecipitation procedure, we studied its applicability with highly purified microbial antigens, the staphylococcal enterotoxins. In addition to their significance in the pathogenesis of disease, these highly purified antigens constitute excellent model proteins for extending the scope of the Farr technique.



## II. MATERIALS AND METHODS

### A. ANTIGENS

Staphylococcal enterotoxin B was obtained from Staphylococcus aureus strain S6, kindly supplied by Dr. F.J. Schantz.<sup>12</sup> This material was purified by chromatography on Amberlite CG-50 resin and carboxymethyl cellulose. It has been characterized as a simple protein with a molecular weight of 35,000. It is a homogeneous material of purity greater than 99%, as indicated by ultracentrifugation, free electrophoresis, and agar gel diffusion tests. Purified staphylococcal enterotoxins A and C were kindly provided by Dr. M.S. Bergdoll, Food Research Institute, University of Wisconsin, Madison. The enterotoxin A was obtained from strain 100 and the enterotoxin C from strain 137. These enterotoxins were also estimated to be at least 99% pure.<sup>13</sup>

### B. ANTISERA

The anti-enterotoxin B reference rabbit antiserum employed (MI-1) was a pool of several sera obtained from five immunized New Zealand rabbits. The anti-enterotoxin B rabbit antisera (MCR) were obtained from five rabbits injected with type B enterotoxoids. A pool of four of these sera (MCR-2) was also employed. The specific anti-enterotoxin B serum (PG-1), used to study both the reproducibility of the test procedure and combination with heterologous enterotoxins, was a pool of 15 sera obtained from immunized rabbits. This serum had a 50% coprecipitation titer of 864 against 1 µg per ml of homologous enterotoxin B. Rabbit antisera to enterotoxins A and C were kindly provided by Dr. M.S. Bergdoll.

### C. GEL DIFFUSION PRECIPITATION TESTS

Double diffusion agar gel precipitation tests were performed by the method of Ouchterlony.<sup>14</sup> Test or reference sera were added to the plates 18 to 24 hours prior to the addition of soluble antigen, and then all plates were incubated at 5 C for at least 72 hours. Single diffusion agar gel precipitation tests were performed by the technique of Oudin<sup>15</sup> as described by Weirether et al.<sup>16</sup> In the Oudin procedure, test sera were evaluated by comparing the migration rate of enterotoxin B in various serum dilutions to migration in similarly diluted MI-1 reference serum. To avoid negative values, the MI-1 serum was arbitrarily given the value of 100. Conventional Ouchterlony block titration of MI-1 serum versus purified enterotoxin B indicated that the maximum sensitivity for detection of antibody was obtained when the concentration of enterotoxin was 10 µg per ml. Oudin block titration of MI-1 serum versus purified enterotoxin B indicated that the maximum sensitivity for detection of antibody was obtained with 25 µg

per ml toxin concentration. Ouchterlony titers cited are the reciprocals of the highest serum dilutions reacting with the standard enterotoxin concentration. Oudin titers represent the antibody content of sera as compared with the value of 100 for the reference MI-1 serum.

#### D. LABELING

Carrier-free iodine<sup>131</sup> was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee. Enterotoxins A, B, and C were iodinated by a microdiffusion procedure previously described.<sup>17</sup> Adequate specific activity was obtained by using 1.0 ml of a solution containing 1 or 2 mg protein per ml, 0.2 ml of 0.002 M KI carrier, and 50 to 100  $\mu$ c of I<sup>131</sup> (as NaI<sup>131</sup>). Free iodine was generated by addition of 0.2 ml of an acidified dichromate solution (0.014 M Na<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> in 1.8 N H<sub>2</sub>SO<sub>4</sub>).

#### E. ANTIBODY DETERMINATION

Antisera for testing were prepared by an initial 1:2.5 dilution of serum, followed by twofold dilutions. All dilutions were made in 0.85% NaCl (saline). To each 0.5-ml sample of diluted serum, 0.5 ml of iodine-labeled antigen was added. Similar control tubes were prepared using the same solutions of labeled antigen and saline. After incubation of antigen-antiserum solutions for 30 minutes, 1 ml of 3.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to each 1 ml antigen-antiserum or antigen-saline solution and immediately mixed. After 18 hours of incubation at 5 C, the tubes were centrifuged for 30 minutes at 2,300 rpm. To avoid nonspecific counts due to adherence of unremoved supernatant fluid to the precipitates or the loss of radioactivity during washing of the precipitates, the nonprecipitated soluble antigen was determined by counts on an accurately measured portion of the supernatant. Sera were usually characterized by one of two alternative methods. In most cases, the dilution of serum giving 50% precipitation of the radioactive antigen was determined by graphic interpolation and taken as the titer. Where indicated, the reciprocal of the highest serum dilution employed giving 50% precipitation of antigen was taken as the titer.

#### F. RADIOACTIVITY MEASUREMENTS

Radioactivity was measured with a Nuclear Chicago Model 132B analyzer-computer in conjunction with a Model DS-302 scintillation well detector containing a 3-inch-diameter sodium iodide crystal. Background radioactivity usually measured less than 50 cpm.

### III. RESULTS

#### A. STUDIES WITH ENTEROTOXINS A, B, AND C

The microdiffusion iodination procedure previously described<sup>17</sup> was employed to label each toxin (1 mg per ml concentration). With this procedure, labeling ratios of 1.7, 1.0, and 1.9 atoms of iodine bound per molecule of enterotoxin A, B, and C, respectively, were obtained. Ouchterlony and Oudin gel diffusion precipitation tests indicated full retention of the precipitating activity of the enterotoxins after iodination and dialysis.

Labeled enterotoxin preparations were stable at 5 C; repeated coprecipitation tests with toxin stored for 2 weeks gave data virtually identical to those obtained with freshly prepared material. Furthermore, essentially all of the iodine precipitated with trichloroacetic acid after storage for 30 days at 5 C. Chromatography by linear gradient elution from carboxymethyl cellulose indicated that enterotoxin B remained essentially homogeneous after iodination.

The solubility of the labeled enterotoxins was determined in various concentrations of ammonium sulfate. The toxins were only slightly soluble in half-saturated (2 M) ammonium sulfate. However, no detectable precipitation occurred in solutions containing 1  $\mu$ g per ml of toxin and 1.6 M ammonium sulfate.

The effects of incubation time and temperature on the interaction between enterotoxin B and antibody were investigated at five time intervals (10, 30, 60, 180 minutes and 24 hours) and two temperatures (5 and 25 C). Dilutions of an antiserum pool (MCR-2) were selected to provide approximately 50% and 100% precipitation of enterotoxin. After the appropriate incubation periods, an equal volume of 3.2 M ammonium sulfate was added. The results indicated that the binding of labeled enterotoxin B by antiserum was rapid, resulting in maximum precipitation after 10 minutes, the shortest interval tested. Similar results were obtained at both incubation temperatures.

The effect of antigen concentration on precipitation was determined in serological tests performed with labeled enterotoxin B concentrations of 10, 2, and 1  $\mu$ g per ml. At the lower concentrations of antigen, essentially 100% of the toxin was precipitated by the immune serum (Fig. 1). Serum titer was taken as the reciprocal of the serum dilution causing precipitation of 50% of the labeled toxin antigen. As Figure 1 indicates, serum titers of 16, 60, and 104 were obtained with antigen concentrations of 10, 2, and 1  $\mu$ g per ml, respectively. As reported previously for the anthrax system,<sup>11</sup> the serum titer of immune serum appeared inversely related to the concentration of enterotoxin B employed.

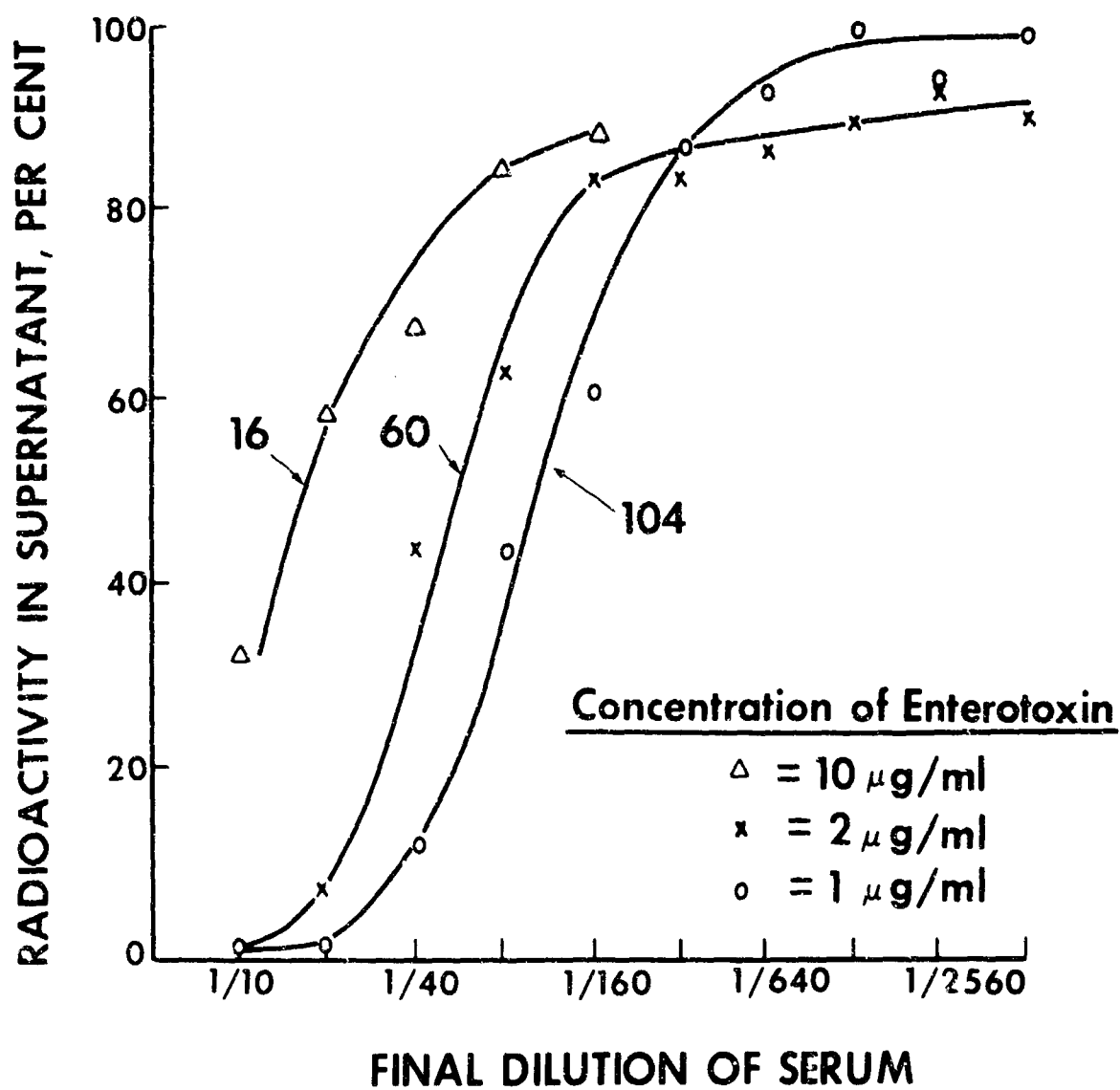


FIGURE 1. Effect of Enterotoxin B Concentration on Coprecipitation with Immune Serum.

To study the reproducibility of the test system, a pooled rabbit anti-serum (PG-1) prepared against enterotoxin B was assayed on three occasions with two independent assays on each occasion. The serum was tested at 10 dilutions, 1:10 through 1:5120, and the labeled toxin was used at a concentration of 1  $\mu$ g per ml. Coprecipitation in all tests was performed with ammonium sulfate at 1.6 M final concentration. The data obtained for the six trials were essentially identical. Fifty per cent precipitation titers ranged from 736 to 992, with a mean value of 864 and a standard deviation of 108.

A variety of rabbit antisera were tested for their capacity to combine with radiolabeled enterotoxins A, B, and C. The toxins were employed at 1  $\mu$ g per ml final concentration, and coprecipitation was performed with 1.6 M ammonium sulfate. Differences were found in the combining capacities of normal and immune rabbit sera for the respective toxins. Approximately 25% or less of the toxins was precipitated by 1:10 diluted normal sera, while similarly diluted immune sera precipitated more than 90% of their homologous toxins (Fig. 1 to 3). With all systems, over a considerable range of dilutions of normal and immune sera, the proportion of antigen precipitated by immune sera remained markedly greater. The combining capacities of normal and immune rabbit sera for the toxin antigens are presented in Table 1. The antisera to the enterotoxins combined with their homologous toxins to a considerable extent. Both normal and BSA immune sera failed to combine with the labeled enterotoxins. The reactions of the anti-B serum (PG-1) with the A and C enterotoxins were greater than anticipated.

## B. COMPARISONS WITH OTHER METHODS

### 1. Quantitative Precipitin Test

Precipitin test data and three pools of rabbit antisera to enterotoxin B were kindly provided by Dr. S.J. Silverman of Fort Detrick. Ammonium sulfate coprecipitation tests were performed with purified enterotoxin B at a final concentration of 1  $\mu$ g per ml. Coprecipitation titers obtained and the resulting comparison are given in Table 2. The results are suggestive of a correlation between coprecipitin test titer and antibody nitrogen content.

### 2. Gel Diffusion Tests

The sensitivity of coprecipitation serology relative to conventional agar gel diffusion procedures was studied in determining antibody to formalin-treated enterotoxin B. Sera obtained from five immunized rabbits at seven different time periods were assayed for coprecipitation antibody using 1.6 M ammonium sulfate for precipitation and labeled enterotoxin B. The results are presented in Table 3. These data indicate that the coprecipitation technique was capable of detecting antibody produced in the rabbit after injection of formalin-treated enterotoxin B. Following a single stimulation, this antibody appeared in low titer and persisted for only a short time. A booster injection elicited a secondary response, producing antibody of high titer which persisted for a longer time. A second booster produced a response of similar magnitude.

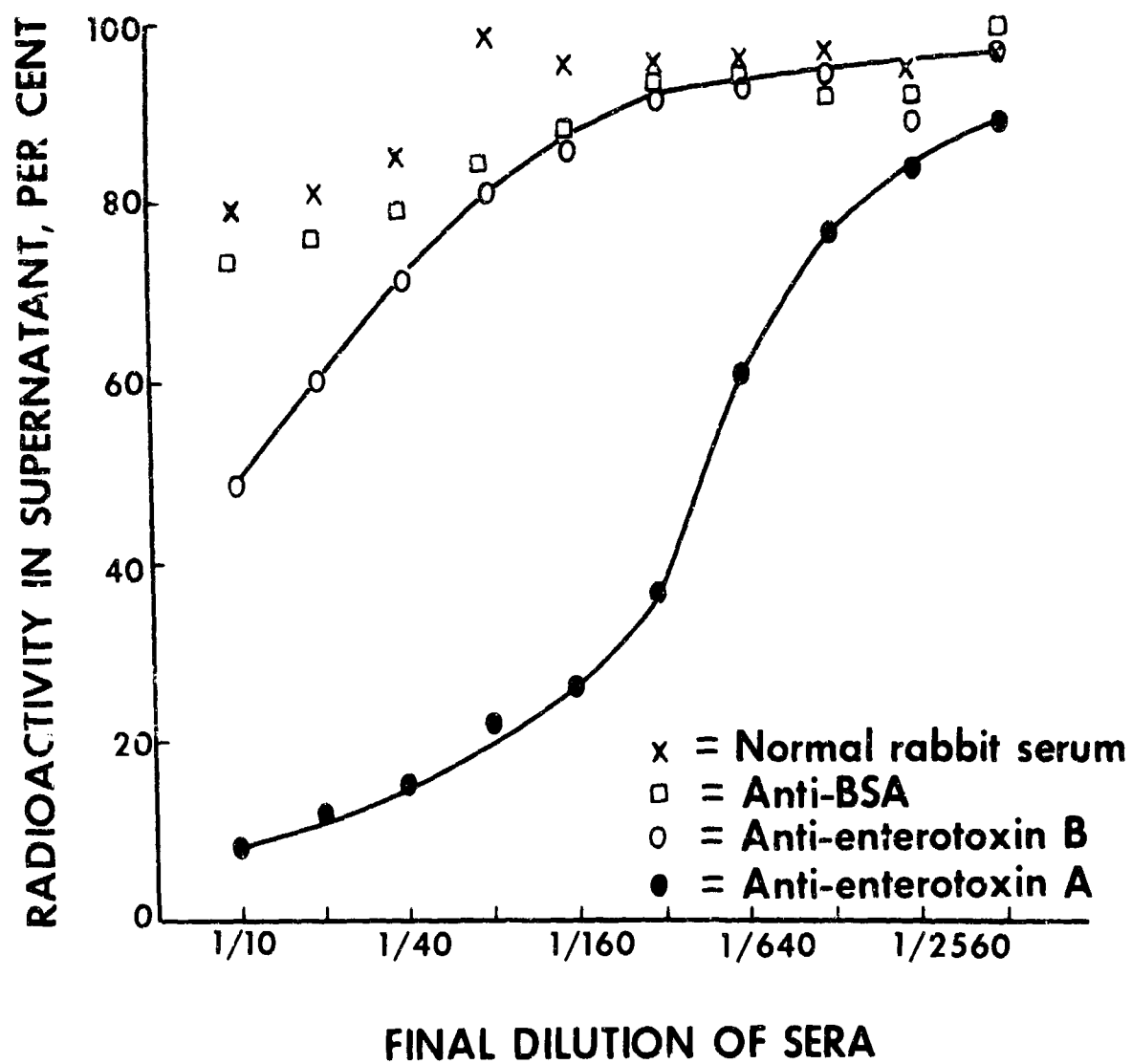


FIGURE 2. Coprecipitation of Labeled Enterotoxin A and Rabbit Sera with 1.6 M Ammonium Sulfate.

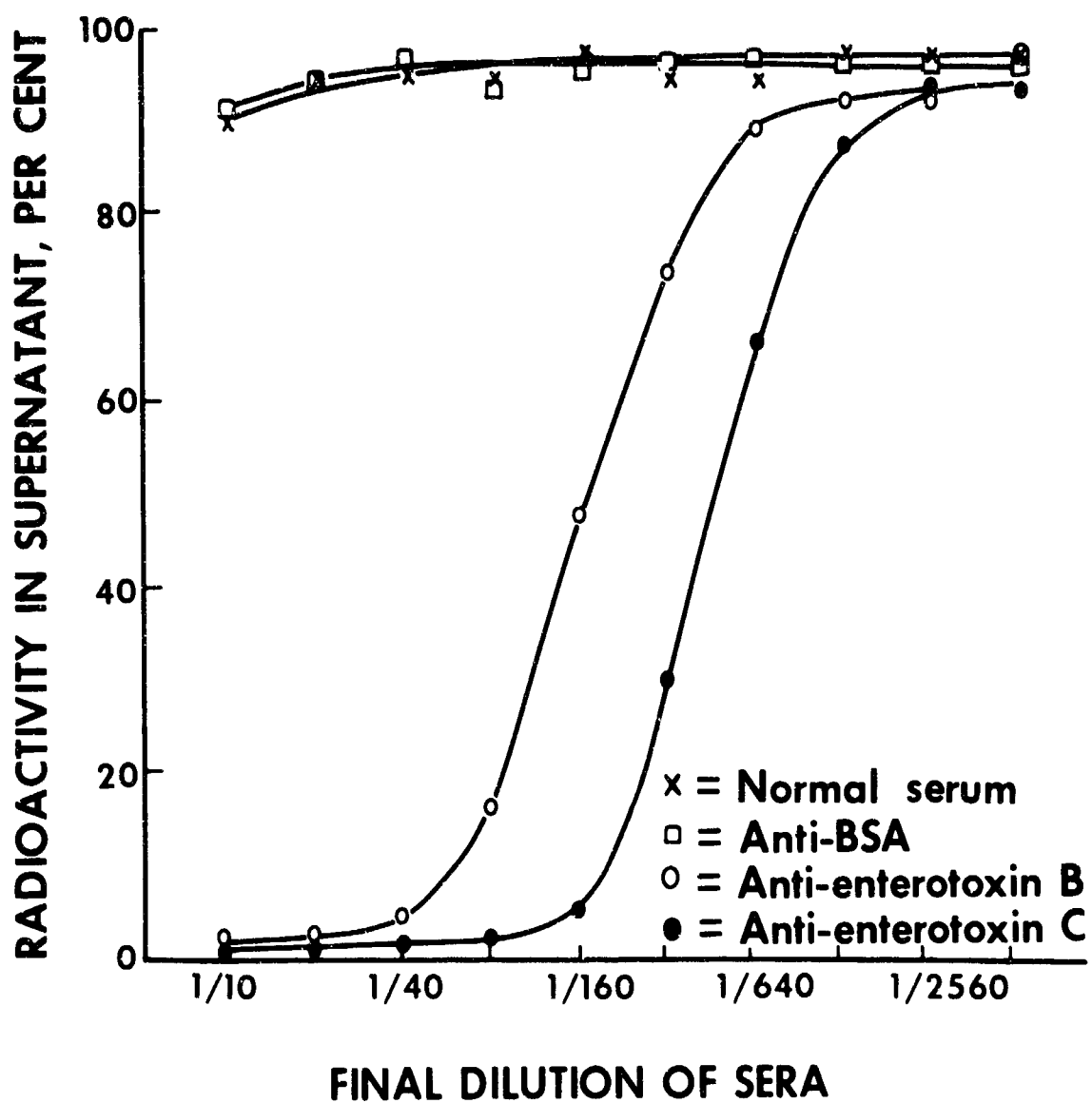


FIGURE 3. Coprecipitation of Labeled Enterotoxin C and Rabbit Sera with 1.6 M Ammonium Sulfate.

TABLE 1. ANTIBODY TO STAPHYLOCOCCAL ENTEROTOXINS  
A, B, AND C IN RABBIT SERA

Serum	50% Titer		
	A Toxin	B Toxin	C Toxin
Normal IV	0	0	-
Normal VI	0	0	0
Anti-bovine albumin	0	0	0
Anti-A toxin	480	-	-
Anti-B toxin	10	864	160
Anti-C toxin	-	-	480

TABLE 2. COMPARATIVE QUANTITATIVE PRECIPITIN AND AMMONIUM SULFATE  
COPRECIPITATION ASSAYS OF ANTI-ENTEROTOXIN B RABBIT SERA

Pool of Serum	Coprecipitation 50% End Point	$\mu$ g Antibody N per ml	Ratio
I	448	230	1.95
II	480	204	2.35
III	384	196	1.96

TABLE 3. DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B ANTIBODY  
BY COPRECIPITATION WITH LABELED TOXIN<sup>a</sup>

Status	Antibody Titers <sup>b</sup> / of Rabbit Number				
	3	5	7	10	15
Preinjection	0	0	0	0	0
Postinjection; 1 month	10	20	0	20	0
Postinjection; 3 months	0	0	0	0	0
Postinjection; 5 months	0	0	0	0	0
Postbooster 1; 5 days	80	320	160	160	160
Postbooster 1; 3 months	10	10	0	10	0
Postbooster 2; 9 days	40	320	160	80	160

a. Iodinated enterotoxin antigen used at a final concentration of 1  $\mu$ g per ml.

b. Titer is the reciprocal of the highest serum dilution used causing precipitation of 50% of the radioactive antigen.



Both Ouchterlony and Oudin tests of these sera detected precipitating antibody only in the sera obtained shortly after booster injections. Both techniques failed to detect response to the initial injection of antigen, or of the antibody persisting after booster injection. Figure 4 illustrates the relationship between coprecipitation determinations and the gel diffusion results. No serum that was positive by a gel diffusion method was negative by coprecipitation. Of the 16 sera that contained antibody by coprecipitation, six (37.5%) were negative by the gel methods.

### C. STUDIES WITH HUMAN SERA

The sera from 53 normal, healthy, human subjects were tested by the coprecipitation technique for antibody to enterotoxin B. All of the sera precipitated measurable amounts of labeled toxin (1  $\mu$ g per ml concentration). Because the combining power of these sera was low, they were characterized by the per cent of antigen precipitated with serum diluted 1:10. Precipitation ranged from 2.8% to 98.8%, with a mean value of 33.2%. The range and distribution of the precipitation values obtained are illustrated in Figure 5. More than half the sera precipitated antigen in the 11 to 20 and 21 to 30% ranges. However, there was a skew to the higher per cent ranges. Twenty of the 53 sera precipitated more than the mean value, and eight of these precipitated more than 50% of the toxin.

Four of the normal sera were also tested for antibody to enterotoxins A and C. All precipitated measurable amounts of both the A and C toxins (Table 4). There was no evident relationship between the combining power for the three toxins.

A group of five individuals studying isolation of enterotoxin B by column chromatography were presumably exposed to enterotoxin during normal laboratory activities. The exposure was apparently minimal, with symptoms limited to lachrymation, conjunctivitis, and periorbital distress. This accident afforded an opportunity to study the antibody response of human subjects following exposure to enterotoxin B. The exposed individuals were bled at the time of the incident and again 2 weeks later. These sera were designated "acute" and "postexposure," respectively. From an existing serum bank, a serum specimen of each individual (obtained at least 2 months prior to the incident) was also obtained and these were designated as "preexposure." Serum antibody to enterotoxin B was measured by coprecipitation serology using purified toxin at 1  $\mu$ g per ml concentration. The results are presented in Table 5. All of the sera precipitated the test antigen to some extent. The sera from three of the subjects (E.L.C., L.L.D., D.P.H.) showed a rise in antigen-binding capacity from the acute to postexposure periods. Two of these three (L.L.D., D.P.H.), which had the highest pre-exposure antibody, also had the highest postexposure levels. These two individuals with high preexposure antibody levels exhibited the mildest clinical symptoms with no eyelid or periorbital edema.

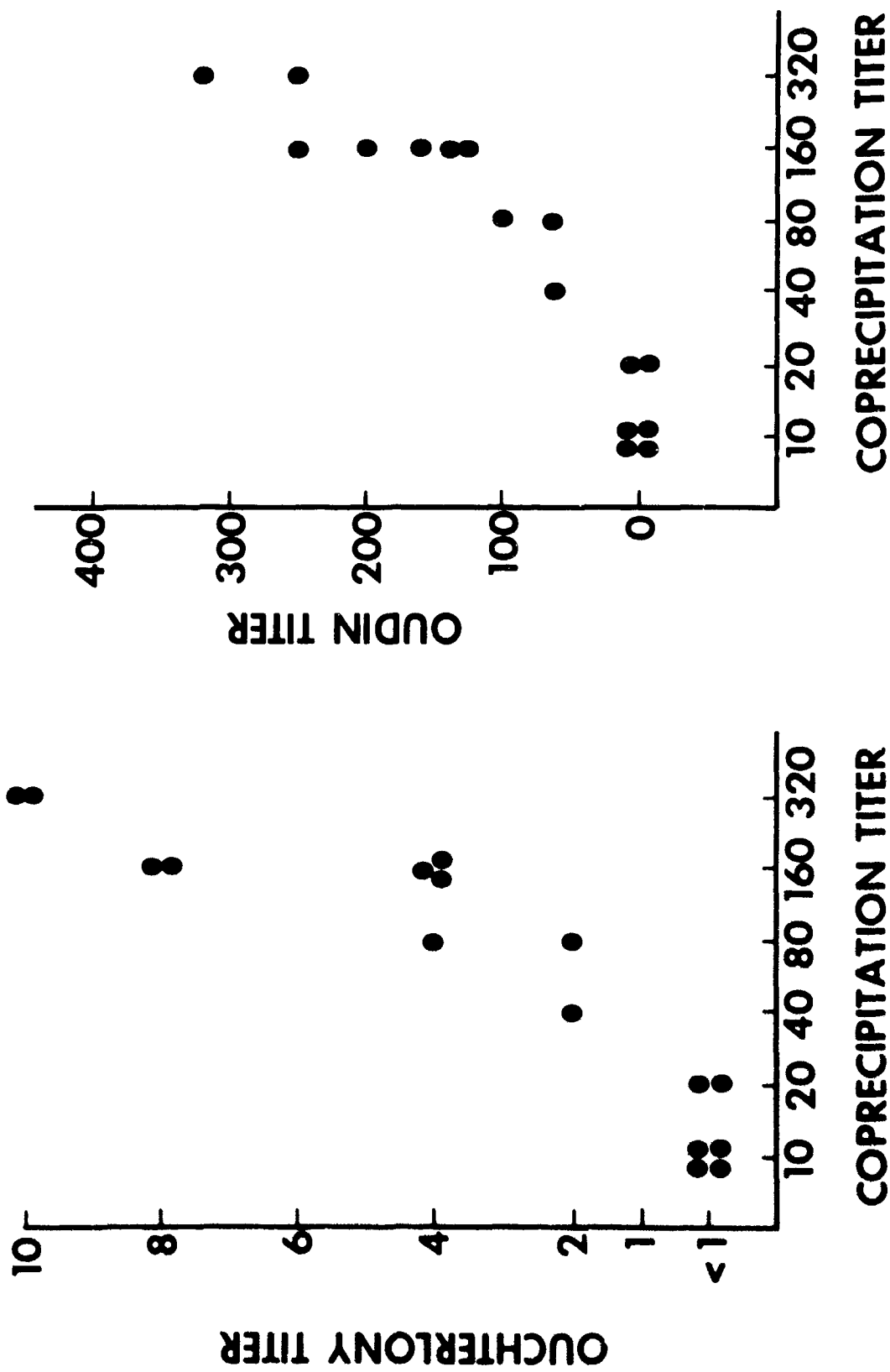


FIGURE 4. Comparison Between Coprecipitation and Gel Diffusion Titers.

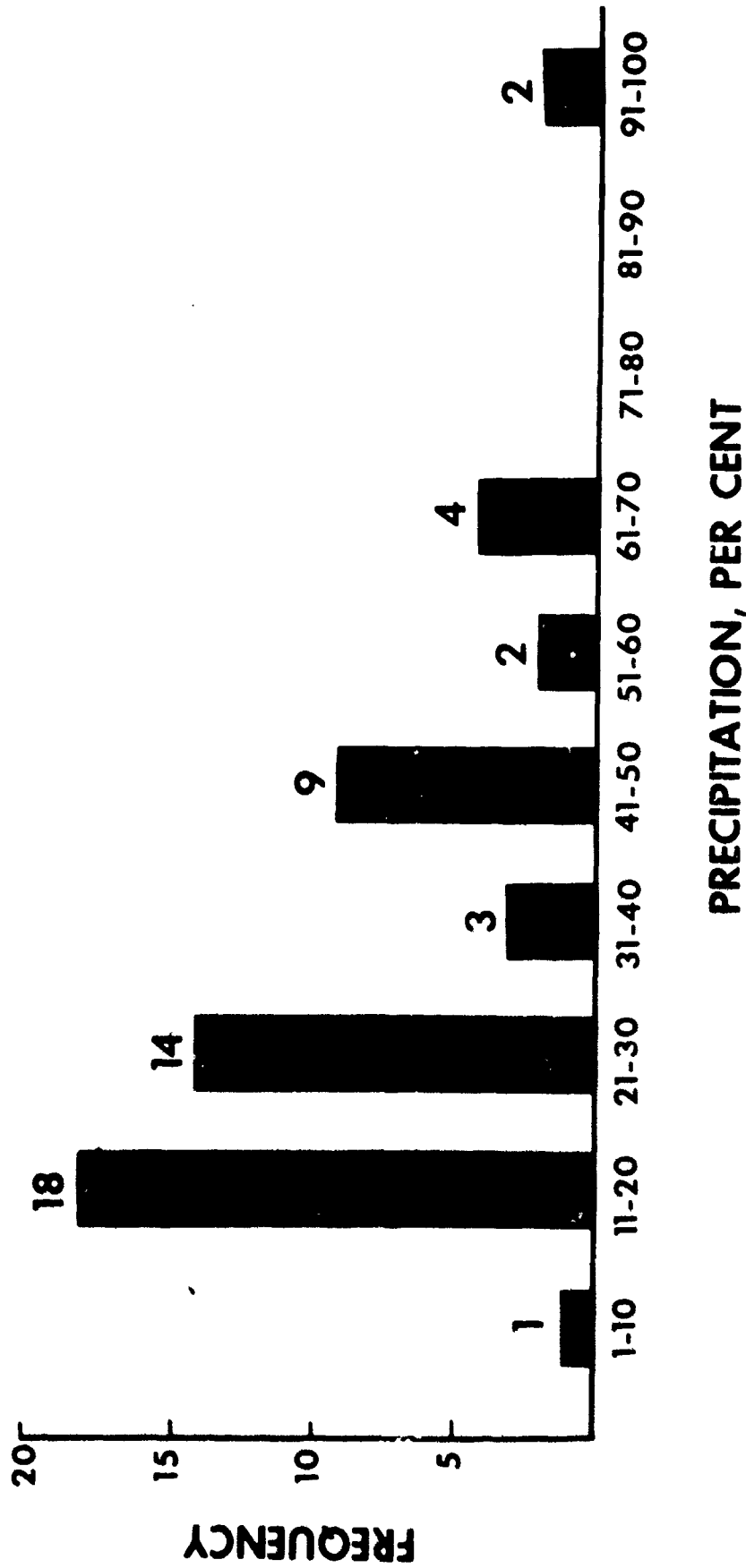


FIGURE 5. Distribution of Coprecipitation Values Obtained with Normal Human Sera.

TABLE 4. ANTIBODY TO STAPHYLOCOCCAL ENTEROTOXIN  
A, B, AND C IN NORMAL HUMAN SUBJECTS

Subject	Precipitation, % <sup>a</sup> /		
	A Toxin	B Toxin	C Toxin
MIK-1	49.7	66.0	21.6
MIK-3	30.2	16.1	10.7
MIK-7	46.3	45.1	14.0
MIK-32	67.1	13.8	-b/

a. Sera at 1:10 dilution.

b. Not done.

TABLE 5. PRECIPITATION OF LABELED STAPHYLOCOCCAL  
ENTEROTOXIN B BY SERA  
OF EXPOSED HUMAN SUBJECTS

Subject	Precipitation, % <sup>a</sup> /		
	Preexposure	Acute	Postexposure
E.L.C.	14.1	19.0	28.8
L.L.D.	28.6	23.8	36.6
D.P.H.	24.9	34.6	43.9
R.H.	16.4	14.8	13.5
W.T.	19.7	19.2	16.3

a. Sera at 1:10 dilution.

#### IV. DISCUSSION

Application of Farr's procedure to microbial antigens requires careful selection of an appropriate concentration of ammonium sulfate. Farr used solubility in half-saturated ammonium sulfate to separate free from bound antigen.<sup>1</sup> Freter<sup>18</sup> and Grey,<sup>10</sup> by altering the concentration of ammonium sulfate employed in the procedure, also obtained systems in which unbound test antigen did not precipitate with globulin. Freter used 37% saturated ammonium sulfate with vibrio antigen, and Grey found it necessary to employ 40% saturation with streptococcal M protein. In the present work, the purified staphylococcal enterotoxins were relatively insoluble at half-saturation, but satisfactory and consistent results were obtained with 1.6 M ammonium sulfate (40% saturation).

In this study purified enterotoxins were employed to assay serum for antibody to enterotoxin. Few reports appear on the in vitro characterization of normal or immune sera employing purified enterotoxin as test antigen. In vivo neutralizing antibodies have been demonstrated in the sera of immunized animals.<sup>19</sup> These antibodies also have been associated with in vitro precipitation of enterotoxin in agar.<sup>20</sup> However, gel diffusion and other serological procedures have been employed primarily for the assay of enterotoxin.<sup>21-23</sup>

Silverman,<sup>21</sup> in assaying culture filtrates for enterotoxin B, found an excellent correlation between the results of gel diffusion precipitation and quantitative precipitin tests. In this study, in determining antibody to enterotoxin B, a similar relationship was suggested between the results of the quantitative precipitin test and ammonium sulfate coprecipitation. Although a relationship was evident between the results of coprecipitation serology and the agar gel diffusion procedures, the coprecipitation procedure was markedly more sensitive.

This sensitivity of the coprecipitation procedure has been demonstrated previously in a study employing BSA.<sup>7</sup> The coprecipitation technique was more sensitive than gel diffusion and other serological procedures tested. The present report indicates that the coprecipitation technique was also more sensitive than gel diffusion methods in the detection of antibody to staphylococcal enterotoxins. Low levels of antibody to enterotoxin B could be detected by ammonium sulfate coprecipitation, while both Ouchterlony and Oudin techniques failed to do so (Fig. 4). Furthermore, with antisera obtained after a booster injection of toxoid, 50% coprecipitation titers could be determined with sera diluted as much as 1:320, while detection of antibody in the same serum by the Ouchterlony technique was limited to dilution of no more than 1:10 (Table 3). Additionally, while the titration of enterotoxin B antibody by Ouchterlony and Oudin tests required 10 µg and 25 µg per ml concentrations of toxin, respectively, the ammonium sulfate coprecipitation test required no more than a 1 µg per ml concentration of antigen.

Antigenic specificity is the basis for establishing the enterotoxin types.<sup>24</sup> The coprecipitation antigen binding tests with the three enterotoxins provide additional evidence regarding the specificity of the antigenic types (Table 1). Specific hyperimmune anti-enterotoxin B serum was not as effective in binding either enterotoxin A or C as it was in binding its homologous toxin. However, it is evident that heterologous interactions also may be significant. Thus, the type B antiserum had considerable combining power for type C toxin, and the reaction involved all of the iodinated protein present in the test antigen and not some heterologous toxin contaminating the preparation (Fig. 3). The data appear consistent with the concept that similar antigenic determinant groups are present on the three toxins. Similar heterologous combining reactions have been described in other systems.<sup>10,25</sup>

The sera of all of the normal human subjects tested contained measurable antigen-binding capacity for purified enterotoxin B (Fig. 5). That this coprecipitating antibody is specific in nature for enterotoxin B is indicated by the markedly different precipitation values obtained when several of these sera were also tested with enterotoxins A and C (Table 4). The presence of these antibodies may indicate some natural exposure to all three enterotoxins.

The ability of the coprecipitation procedure to determine antibody in all of the human subjects surveyed indicates that this serological technique can be useful in following humoral response to enterotoxin stimulation. Further support for this view is found in the data obtained with the accidentally exposed human subjects (Table 5). At every test period, each of the subjects' sera demonstrated some antigen-binding capacity. Three of the five subjects examined presented clear evidence of a rise in coprecipitating antibody following exposure. Additionally, the relationship between clinical symptoms and titer of preexposure antibody is the sort that would be expected if the antigen-binding capacity of serum were related to protection of the host.

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DOCUMENT CONTROL DATA - R & D		
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)		
1. ORIGINATING ACTIVITY (Corporate author)		26. REPORT SECURITY CLASSIFICATION
Department of the Army Fort Detrick, Frederick, Maryland, 21701		Unclassified
2. REPORT TITLE		27. GROUP
AMMONIUM SULFATE COPRECIPITATION ANTIBODY DETERMINATION WITH PURIFIED STAPHYLOCOCCAL ENTEROTOXINS		
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)		
5. AUTHOR(S) (First name, middle initial, last name)		
Jack (NMI) Gruber George G. Wright		
6. REPORT DATE	7A. TOTAL NO. OF PAGES	7B. NO. OF REFS
January 1969	25	25
8A. CONTRACT OR GRANT NO.		8B. ORIGINATOR'S REPORT NUMBER(S)
A. PROJECT NO. 1B061102B71A		Technical Manuscript 491
C.		9B. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
D.		
10. DISTRIBUTION STATEMENT		
Qualified requesters may obtain copies of this publication from DDC. Foreign announcement and dissemination of this publication by DDC is not authorized. Release or announcement to the public is not authorized.		
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY
		Department of the Army Fort Detrick, Frederick, Maryland, 21701
13. ABSTRACT		
<p>The ammonium sulfate coprecipitation technique of Farr was applied to purified enterotoxins of <u>Staphylococcus aureus</u>. Studies with iodine<sup>131</sup>-labeled enterotoxins A, B, and C, using 1.6 M ammonium sulfate for coprecipitation, revealed differences in the antigen-binding capacity of normal and immune rabbit sera for the enterotoxins. The ammonium sulfate coprecipitation procedure was more sensitive than agar gel diffusion methods for the determination of antibody to enterotoxin B. Antigen-binding tests with anti-enterotoxin B rabbit serum suggest the presence of similar antigenic determinant groups in all three toxins. Measurable antigen-binding capacities for enterotoxins A, B, and C were detected in sera of normal human subjects. Increased levels of binding antibody to enterotoxin B were detected in three of five accidentally exposed human subjects.</p>		
14. Key Words		
<p>*Ammonium sulfate coprecipitation technique *Antibodies *Staphylococcal enterotoxin Iodine 131 *Antigens Antiserums Immunology</p>		

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